### Coverslips and Imaging Chambers

As for many other light microscopy techniques, **#1.5** or **#1.5H glass coverslips** (thickness: 170 μm) are required in all cases. Many imaging dishes and chambered slides are also available with #1.5H glass bottoms.

→ **Do not** use plastics coverslips or coverslips with a different thickness.

→ Please **avoid gridded coverslips**, as the grooves may affect the focus.

### Background and Brightness of the Samples

In particular, background labeling and brightness of the samples are of special importance for *MINFLUX*.

*MINFLUX* is a single molecule localization technique, so every photon of background is reducing the information collected from your fluorophore. This means a sample which is geared towards ultimate brightness is **not** **important** - but to work towards a **background-free sample**.

To create such a sample, it is advisable to **minimize the amount of excess or non-specific fluorophore in the sample**. You may find that you need to increase your blocking step incubation times, increase the number and length of wash steps, or reduce your antibody concentration. Quenching of your sample, for example by **ammonium chloride, glycine, or sodium borohydride,** may also be incorporated where aldehyde fixation protocols are used.

## Gold Nano Particles

We recommend to use the following gold nano particles / gold beads for the IR reflection based stabilization system and also the MBM stabilization system. The correct type of gold beads is especially important when using MBM for best stabilization against drift during acquisition of *MINFLUX* images. Gold nano rods for example don't give an even shaped reflection and therefore are not suitable for MBM measurements. Gold fuducials included in the glas surface (e.g. coverslips from Hestzig) give no signal that is useable for MBM.

* BBI solutions Gold Colloid 150nm Cat#EM.GC.150/4 20ml www.bbisolutions.com
* Nanopartz 200nm gold nanoparticles Cat#A11-200-CIT-DIH-1-10 10ml. www.nanopartz.com

As the beads age over time it is not recommended to order more beads than can be used in about 3-6 month. The beads typically become more shaky the older they are.

Note: reflection properties of gold nano particles depend on size and type. MBM of delivered *MINFLUX* microscopes are setup and tested on 150nm BBI gold beads. If other beads are used the MBM laser power setting might need to be adjusted. If you feel this is necessary for your system, please get in touch with the Abberior Support or Application team

### DNA-PAINT

DNA-PAINT uses binding kinetics of short DNA oligomers rather than manipulating photophysics to generate the ‘blinks’ at the labelled epitope. The protein of interest is labelled with a docking strand, with the complementary ‘imager’ oligomer labeled with a fluorophore binding and unbinding over short timescales. The imager fluorophores (640 nm: ATTO 655, *abberior RED*; 561 nm: Cy3B, *abberior ORANGE*) should be bright and photostable – avoiding blinking dyes and conditions under which dyes can show a blinking behavior.

Sequential multi-color imaging is also possible using exchange-PAINT. Samples are labelled with different docking/imager strand pairs for each epitope. Imager strands are exchanged and images are acquired sequentially whilst maintaining the same reference position in the *MINFLUX* stabilization system.

*MINFLUX* PAINT

For PAINT acquisitions, it is important to optimize your imager strand concentration. More imager strand will result in more frequent ‘blinks’, but can also result in too many fluorescing molecules in close proximity. High imager strand concentrations can also significantly increase background, as the fluorophore will still fluoresce when in the buffer unbound. Adjusting the imaging buffer composition and the docking/imager strand pairs can also affect the length of time the imager strand remains bound, and thereby regulate imaging speed (Schueder et al., Nat Methods, 2019).

Aim for an appropriate blinking density and background for *MINFLUX* while not overly reducing imaging speed.

## Sample Storage

Prior to imaging, fixed samples can be stored in washing buffer (e.g. PBS) at 4 °C, as for standard immunofluorescence samples. Gold fiducials should be added to the sample before mounting. Previously mounted samples can be stored at 4 °C for short periods (< 5 days) before remounting and reimaging, but fiducial stability can be reduced with time. For immunolabeled samples, especially with single domain antibodies, consider post-fixation to prolong sample stability.